Protocol for PCR
With Taq

Avoiding Contamination
PCR allows the production of more than 10 million copies of a target DNA sequence from only a few molecules. The sensitivity of this technique means that the sample should not be contaminated with any other DNA or previously amplified products (amplicons) that may reside in the laboratory environment.

• DNA sample preparation, reaction mixture assemblage and the PCR process, in addition to the subsequent reaction product analysis, should be performed in separate areas.
• A Laminar Flow Cabinet equipped with a UV lamp is recommended for preparing the reaction mixture.
• Fresh gloves should be worn for DNA purification and each reaction set-up.
• The use of dedicated vessels and positive displacement pipettes or tips with aerosol filters for both DNA sample and reaction mixture preparation, is strongly recommended.
• The reagents for PCR should be prepared separately and used solely for this purpose. Autoclaving of all solutions, except dNTPs, primers and Taq DNA Polymerase is recommended. Solutions should be aliquoted in small portions and stored in designated PCR areas. Aliquots should be stored separately from other DNA samples.
• A control reaction, omitting template DNA, should always be performed, to confirm the absence of contamination.

Preparation of Reaction Mixture
To perform several parallel reactions, prepare a master mix containing water, buffer, dNTPs, primers and Taq DNA Polymerase in a single tube, which can then be aliquoted into individual tubes. MgCl2 and template DNA solutions are then added. This method of setting reactions minimizes the possibility of pipetting errors and saves time by reducing the number of reagent transfers.

Reaction Mixture Set Up
1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Add, in a thin-walled PCR tube, on ice:

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
<th>Quantity for 50 µl of Rxn</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile deionized water</td>
<td>-</td>
<td>Variable</td>
</tr>
<tr>
<td>10X Taq buffer</td>
<td>1 X</td>
<td>5 µl</td>
</tr>
<tr>
<td>2mM dNTP mix</td>
<td>0.2mM of each</td>
<td>5 µl</td>
</tr>
<tr>
<td>Primer I *</td>
<td>0.1-1µM</td>
<td>Variable</td>
</tr>
<tr>
<td>Primer II *</td>
<td>0.1-1µM</td>
<td>Variable</td>
</tr>
<tr>
<td>Taq DNA Polymerase</td>
<td>1.25u/50µl</td>
<td>Variable</td>
</tr>
<tr>
<td>25mM MgCl2</td>
<td>1 -4 mM</td>
<td>Variable</td>
</tr>
<tr>
<td>Template DNA</td>
<td>10pg-1µg</td>
<td>Variable</td>
</tr>
</tbody>
</table>

* or about 50 pmol = 16.3ng X # of bp
3. Gently vortex the sample and briefly centrifuge to collect all drops from walls of tube.
4. Place samples in a thermocycler and start PCR.

Components of the Reaction Mixture

Template DNA. Usually the amount of template DNA is in the range of 0.01-1ng for plasmid or phage DNA and 0.1-1µg for genomic DNA, for a total reaction mixture of 50µl. Higher amounts of template DNA usually increase the yield of nonspecific PCR products, but if the fidelity of synthesis is crucial, maximal allowable template DNA quantities together with a limited number of PCR cycles should be used to increase the percentage of "correct" PCR products. Nearly all routine methods are suitable for template DNA purification. Although even trace amounts of agents used in DNA purification procedures (phenol, EDTA, Proteinase K, etc.) strongly inhibit Taq DNA Polymerase, ethanol precipitation of DNA and repetitive treatments of DNA pellets with 70% ethanol is usually effective in removing traces of contaminants from
Protocol for PCR

With Taq

the DNA sample.

**Primers. Guidelines for primer selection:**

PCR primers are usually 15-30 nucleotides in length. Longer primers provide higher specificity.

- The GC content should be 40-60%. The C and G nucleotides should be distributed uniformly throughout of the primer. More than three G or C nucleotides at the 3’-end of the primer should be avoided, as nonspecific priming may occur.
- The primer should not be self-complementary or complementary to any other primer in the reaction mixture, in order to avoid primer-dimer and hairpin formation.
- The melting temperature of flanking primers should not differ by more than 5°C, so the GC content and length must be chosen accordingly.
- All possible sites of complementarity between primers and the template DNA should be noted.
- If primers are degenerate, at least 3 conservative nucleotides must be located at the primer’s 3’-end.
- Estimation of the melting and annealing temperatures of primer:

  If the primer is shorter than 25 nucleotides, the approx. melting temperature (Tm) is calculated using the following formula:

\[
Tm = 4(G+C) + 2(A+T)
\]

- G, C, A, T - number of respective nucleotides in the primer.
- Annealing temperature should be approx. 5°C lower than the melting temperature.
- If the primer is longer than 25 nucleotides, the melting temperature should be calculated using specialized computer programs where the interactions of adjacent bases, the influence of salt concentration, etc., are evaluated.

**MgCl₂ Concentration.**

Since Mg²⁺ ions form complexes with dNTPs, primers and DNA templates, the optimal concentration of MgCl₂ has to be selected for each experiment. Too few Mg²⁺ ions result in a low yield of PCR product, and too many increase the yield of non-specific products and promote misincorporation. Lower Mg²⁺ concentrations are desirable when fidelity of DNA synthesis is critical. The recommended range of MgCl₂ concentration is 1-4mM, under the standard reaction conditions specified. In our experiments, at a final dNTP concentration of 0.2mM, a MgCl₂ concentration ranges of 1.5±0.25mM (in Taq buffer with KCl) and of 2.0±0.5mM (in Taq buffer with (NH₄)₂SO₄) are suitable in most cases. If the DNA samples contain EDTA or other chelators, the MgCl₂ concentration in the reaction mixture should be raised proportionally.

**dNTPs.**

The concentration of each dNTP in the reaction mixture is usually 200µM. It is very important to have equal concentrations of each dNTP (dATP, dCTP, dGTP, dTTP), as inaccuracy in the concentration of even a single dNTP dramatically increases the misincorporation level.

- When maximum fidelity of the PCR process is crucial, the final dNTP concentration should be 1050µM, since the fidelity of DNA synthesis is maximal in this concentration range. In addition, the concentration of MgCl₂ should be selected empirically, starting from 1mM and increasing in 0.1mM steps, until a sufficient yield of PCR product is obtained.

**Taq DNA Polymerase.**

Usually 1-1.5u of Taq DNA Polymerase are used in 50µl of reaction mix. Higher Taq DNA Polymerase concentrations may cause synthesis of nonspecific products. However, if inhibitors are present in the reaction mix (e.g., if the template DNA used is not highly purified), higher amounts of Taq DNA Polymerase (2-3u) may be necessary to obtain a better yield of amplification products.

March 05
Protocol for PCR

With Taq

Reaction Overlay.

If necessary, the reaction mixture can be overlaid with mineral oil or paraffin (melting temperature 50-60°C) of special PCR grade. One-half of the total reaction volume is usually sufficient.

Cycling Conditions

Amplification parameters depend greatly on the template, primers and amplification apparatus used.

Initial Denaturation Step.
- The complete denaturation of the DNA template at the start of the PCR reaction is of key importance. Incomplete denaturation of DNA results in the inefficient utilization of template in the first amplification cycle and in a poor yield of PCR product. The initial denaturation should be performed over an interval of 1-3 min at 95°C if the GC content is 50% or less. This interval should be extended up to 10 min for GC-rich templates.
- If the initial denaturation is no longer than 3 min at 95°C, Taq DNA Polymerase can be added into the initial reaction mixture. If longer initial denaturation or a higher temperature is necessary, Taq DNA Polymerase should be added only after the initial denaturation, as the stability of the enzyme dramatically decreases at temperatures over 95°C.

Denaturation Step.

Usually denaturation for 0.5-2 min at 94-95°C is sufficient, since the PCR product synthesized in the first amplification cycle is significantly shorter than the template DNA and is completely denatured under these conditions. If the amplified DNA has a very high GC content, denaturation time may be increased up to 3-4 min. Alternatively, additives facilitating DNA denaturation - glycerol (up to 10-15 vol.%), DMSO (up to 10%) or formamide (up to 5%) - should be used. In the presence of such additives, the annealing temperature should be adjusted experimentally, since the melting temperature of the primer-template DNA duplex decreases significantly when these additives are used. The amount of enzyme in the reaction mix should be increased since DMSO and formamide, at the suggested concentrations, inhibit Taq DNA Polymerase by approx. 50%. Alternatively, a common way to decrease the melting temperature of the PCR product is to substitute dGTP with 7-deaza-dGTP in the reaction mix.

Primer Annealing Step.

Usually the optimal annealing temperature is 5°C lower than the melting temperature of primer-template DNA duplex. Incubation for 0.5-2 min is usually sufficient. However, if nonspecific PCR products are obtained in addition to the expected product, the annealing temperature should be optimized by increasing it stepwise by 1-2°C.

Extending Step.

Usually the extending step is performed at 70-75°C. The rate of DNA synthesis by Taq DNA Polymerase is highest at this temperature. Recommended extending time is 1 min for the synthesis of PCR fragments up to 2 kb. When larger DNA fragments are amplified, the extending time is usually increased by 1 min for each 1000 bp.

Number of Cycles.
Protocol for PCR
With Taq

The number of PCR cycles depends on the amount of template DNA in the reaction mix and on the expected yield of the PCR product. For less than 10 copies of template DNA, 40 cycles should be performed. If the initial quantity of template DNA is higher, 25-35 cycles are usually sufficient.

**Final Extending Step.**

After the last cycle, the samples are usually incubated at 72°C for 5-15 min to fill-in the protruding ends of newly synthesized PCR products. Also, during this step, the terminal transferase activity of Taq DNA Polymerase adds extra A nucleotides to the 3' ends of PCR products. Therefore, if PCR fragments are to be cloned into T/A vectors, this step can be prolonged to up to 30 min.